Commentary

A complex network regulating malate contents during fruit ripening in climacteric fruits

Malate is one of the most abundant organic acids in vascular plants and its synthesis takes place in the cytosol, in chloroplasts and in mitochondria, as well as in peroxisomes/glyoxisomes (Fernie & Martinoia, 2009; Sweetman *et al.*, 2009). In mitochondria, malate is a substrate of the Krebs cycle, and in glyoxisomes, it is a metabolite of the glyoxylate cycle. Malate can become oxidized by the NADP malic enzyme leading to the release of NADPH and CO_2, a process that is present in all plants, but of particular importance in C_4 and CAM plants. Apart from this, malate plays an important role as an osmoticum, it serves as an intermediate to shuttle NADH across the plasma and organellar membranes, and it contributes to cellular pH regulation and is a store for CO_2 in CAM plants. Malate levels in the cytoplasm are tightly controlled, ranging between 400 and 800 µM (Winter *et al.*, 1994; Farré *et al.*, 2001). In contrast to these low levels, malate in vacuoles can accumulate at very high concentrations, where up to 200 mM for grapes and apples has been reported (Zhang *et al.*, 2010; Famiani *et al.*, 2016). It was demonstrated that malate concentrations change dependent on the nutritional and metabolic status of a plant. Very strong fluctuations have been reported for CAM plants and guard cells, which attracted much interest in the scientific community (Fernie & Martinoia, 2009; Sweetman *et al.*, 2009). It is textbook knowledge that the maturation of climacteric fruits is initiated by phytohormone ethylene, leading to malate degradation, and a concomitant rise of the pH and sugar production (a process known as 'sweetening'). Unfortunately, the exact interaction between ethylene production and fruit maturation, in terms of malate homeostasis, is unknown. This fact is surprising given that the fruit pH, in conjunction with sugars and secondary compounds, determines the overall taste (Cohen *et al.*, 2014) and therefore has a strong economic impact. However, in an article published in this issue of *New Phytologist,* Wang *et al.* (2023; pp. 1014–1034) used an impressive set of sophisticated experimental approaches to demonstrate that the interaction of two transcription factors, namely the ethylene response factor *Md*ERF72 and the WRKY isoform *Md*WRKY31, plays a central role in regulating malate levels during apple fruit ripening.

As outlined above, malate is stored in vacuoles and this accumulation is mediated by two transport proteins, the tonoplast dicarboxylate transporter (tDT), a homolog of renal carboxylate transporters (Emmerlich *et al.*, 2003), and ALMTs, representing plant specific malate and chloride channels (Kovermann *et al.*, 2007). In search of factors responsible for low acidity in apples, the gene *Ma1* (*Md*ALMT9) was found (Bai *et al.*, 2012), which exerts a marked impact on fruit pH. Accordingly, mutants with defects in *Ma1* exhibited a much higher pH, and a truncated form of *Md*ALMT9 leads to strongly impaired vacuolar malate transport (Li *et al.*, 2020). Independent studies showed that in climacteric fruits several ethylene-induced transcription factors are responsible for fruit softening (Tacken *et al.*, 2010). Thus, the fact that malate contents decrease in response to ethylene during fruit maturation, and that this process is impaired by ethylene synthesis inhibitors, led the authors to look for factors that are responsible for this interaction.

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As a first step, Wang *et al.* performed an RNA-Seq analysis to determine which genes are upregulated when apple fruits are treated with an ethylene inhibitor. Genes-encoding proton pumps, malate transporters, and enzymes involved in malate synthesis, together with two regulatory transcription factors, were found to be upregulated. By contrast, the expression of the malic enzyme gene, which is involved in malate degradation, was downregulated. To find ethylene-responsive elements in these genes, the authors performed promoter analyses and identified an ethylene-responsive element only in *Md*ALMT9. Using a DNA trapping approach, together with electrophoretic mobility shift assays, the transcription factor *Md*ERF72 appeared among the proteins bound to the ethylene-responsive *cis* element. Subsequently, the expression of *Md*ERF72 promoter β-glucuronidase (GUS)- or luciferase (LUC) constructs in apple tissue demonstrated that respective activities increased in the presence of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC).

Similarly, the expression of this transcription factor increased also during maturation, concomitantly with ethylene synthesis. Further experiments confirmed that *Md*ERF72 requires the GCC ethylene-responsive element to bind to the *Md*ALMT9 promoter. To investigate the role of *Md*ERF72 in regulating *Md*ALMT9 the authors expressed the promoter of *Md*ALMT9 fused to GUS and generated calli in which this single construct was expressed or in combination with $35S_{pro}$*::MdERF72.* Calli expressing both constructs exhibited a much lower GUS activity indicating that *Md*ERF72 exhibits a negative effect on *MdALMT9* expression. In

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line with this, the overexpression of *MdERF72* decreased both *MdALMT9* mRNA levels and malate concentrations. Interestingly, downregulation of *MdALMT9* resulted in a downregulation of the ethylene-independent malate synthesis genes, and the upregulation of malic enzyme, but had no effect on vacuolar proton pumps. In summary, these results indicate that malate synthesis is tightly controlled, possibly by a feedback mechanism, to keep cytosolic concentrations of this multifunctional solute balanced.

To identify factors that interact with *Md*ERF72, and by this to participate in the regulation of *Md*ALMT9, a yeast two-hybrid screen, BiMolecular Fluorescence Complementation, and *in vitro* pull-done assays were conducted. The major finding of this concerted approach was the observation that the interaction of *Md*WRKY31 with *Md*ERF72 specifically inhibits the effect of *Md*ERF72 on *MdALMT9* expression. Various experiments in which *MdWRKY31* was overexpressed in fruits or calli of apple revealed that this transcription factor increases both *MdALMT9* transcripts and malate contents. The identification of *Md*WRKY31 binding to a W-box domain in the *MdERF72* promotor explains how *MdERF72* transcript levels are decreased, while in comparison the binding of *Md*WRKY31 to a W-box domain of the *Md*ALMT9 promoters results in stimulation of that transcripts. Hence, the positive effect of *Md*WRKY31 on *Md*ALMT9 and malate contents is due to, first the interaction of *Md*WRKY31 with *Md*ERF72, which reduces the capacity of *Md*ERF72 to inhibit MdALMT9 expression, second the binding to the *Md*ERF72 promoter that inhibits its expression, and lastly the interaction and stimulatory effect on the *Md*ALMT9 promoter. The confirmation of the causality of these interactions *in situ* is provided by the generation of corresponding overexpressor or silencing mutants. These final analyses clearly showed that all molecular components mentioned above are part of a complex ethylene-modulated network controlling apple fruit malate levels and by this fruit pH.

During the last decades, many vacuolar transporters have been characterized and identified (Martinoia, 2018). However, our knowledge about how they are regulated; how they are integrated into the complex transport network between cytosol and vacuole; how they contribute to metabolic homeostasis; and how they respond to changing environmental conditions is still far from complete (Martinoia *et al.*, 2007). Here, the authors provide a very comprehensive and convincing study uncovering molecular details of how malate transport is regulated in climacteric fruits.

This study may help to solve some still open questions, for example, how is malate transport regulated in non-climacteric fruits, like grape berries, where a very similar pattern can be observed for this carboxylic acid during maturation. Does the ethylene peak that has been observed (Chervin *et al.*, 2004) play a similar role in decreasing malate content? Can a similar approach be used to learn more about malate fluctuations in CAM plants and guard cells? The decrease in malate contents cannot be explained solely by decreased vacuolar uptake, and thus, malate must undergo controlled release from the vacuole, so which transporters are responsible for this process? Finally, *At*tDT has been shown to allow malate fluxes in both directions (Frei *et al.*, 2018) and *At*ALMT4 is able to release malate from the vacuole (Eisenach *et al.*, 2017) – is there a continuous release of malate from the vacuole or is this process also induced and regulated? State-of-the-art techniques used in a new context allowed Wang *et al.* to present important information on how vacuolar, and indirectly cytosolic malate concentrations, are regulated. This work is therefore important for apple producers as they can gather new insights into which factors play an important role in modulating apple acidity. More generally, this is a breakthrough that could potentially lead to answers for many open malate questions in the near future.

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